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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US99/19393 <b>(22) International Filing Date:</b> 25 August 1999 (25.08.99) <b>(30) Priority Data:</b> 09/139,902 25 August 1998 (25.08.98) US <b>(71) Applicants:</b> UNIVERSITY OF GEORGIA RESEARCH FOUNDATION, INC. [US/US]; The Boyd Graduate Studies Research Center, Athens, GA 30602-7411 (US). AVIGENICS, INC. [US/US]; 845 Oak Grove Avenue #220, Menlo Park, CA 94025 (US). <b>(72) Inventors:</b> IVARIE, Robert; 54 Jackson Street, Watkinsville, GA 30677 (US). HARVEY, Alex, J.; 188 Sunset Drive, Athens, GA 30606 (US). MURPHY, George, F., Jr.; 1723 Santa Cruz Avenue, Menlo Park, CA 94025 (US). RAPP, Jeffrey, C.; 48 Third Street, Watkinsville, GA 30677 (US). <b>(74) Agents:</b> CHOW, Y., Ping et al.; Heller Ehrman White & McAuliffe, 525 University Avenue, Palo Alto, CA 94301-1900 (US).		<b>(81) Designated States:</b> AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), DM, EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> DIRECT OVIDUCT TRANSGENESIS <b>(57) Abstract</b> <p>Methods for preparing transgenic avians which express exogenous protein substantially only in their oviducts are disclosed. Each of the methods comprises delivering nucleic acid expression cassettes directly to the oviducts of the avians. The exogenous protein expressed by the expression cassette is secreted into the lumen of the avian oviduct and secreted into the eggs of the transgenic avians. Methods for preparing eggs which contain exogenous protein, such as human interferon, and methods for the production of proteins are also disclosed. The methods for direct oviduct transgenesis may also be used to assess the suitability of expression cassettes or exogenous proteins for expression in the avian oviduct.</p>		

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## DIRECT OVIDUCT TRANSGENESIS

### BACKGROUND OF THE INVENTION

5

#### a) Field of the Invention

This invention relates generally to methods of producing transgenic birds. In particular, the invention relates to methods of delivering a transgene directly to the avian oviduct to produce a bird which expresses an exogenous protein in its  
10 avian oviduct and deposits that exogenous protein into its eggs.

#### a) Description of Related Art

Numerous natural and synthetic proteins are used in diagnostic and therapeutic applications; many others are in development or in clinical trials.  
15 Current methods of protein production include isolation from natural sources and recombinant production in bacterial and mammalian cells. Because of the complexity and high cost of these methods of protein production, however, efforts are underway to develop alternatives. For example, methods for producing exogenous proteins in the milk of pigs, sheep, goats and cows have been reported.  
20 These approaches suffer from several limitations, including long generation times between founder and production transgenic herds, extensive husbandry and veterinary costs, and variable levels of expression because of position effects at the site of the transgene insertion in the genome. Proteins are also being produced using milling and malting processes from barley and rye. However, plant post-  
25 translational modifications differ from vertebrate post-translational modifications, which often has a critical effect on the biological activity of the exogenous proteins.

The avian reproductive system, including that of the chicken, is well described. The egg of the hen consists of several layers which are secreted upon  
30 the yolk during its passage through the oviduct. The production of an egg begins

with formation of the large yolk in the ovary of the hen. The unfertilized oocyte is positioned on top of the yolk sac. Upon ovulation or release of the yolk from the ovary, it passes into the infundibulum of the oviduct where it is fertilized if sperm are present. It then moves into the magnum of the oviduct which is lined with  
5 tubular gland cells. These cells secrete the egg-white proteins, including ovalbumin, lysozyme, ovomucoid, conalbumin and ovomucin, into the lumen of the magnum where they are deposited onto the avian embryo and yolk.

The hen oviduct is an excellent organ for a protein bioreactor because of high levels of protein production, proper folding and post-translation modification  
10 of the target protein, and ease of product recovery. As a result, efforts have been made to create transgenic chickens expressing exogenous proteins in the oviduct (see PCT publication WO 97/47739). However, the production of transgenic chickens can be both time-consuming and inefficient. In fact, a key limitation of using any animal as a bioreactor is the time required (approximately 9 months for  
15 chickens, 2-3 years for ungulates) to introduce the desired transgene into the animal's genome.

Because of the nature of the pharmaceutical and biotechnology industries, achieving gram quantities of protein production in 6 months or less is very desirable. A short turn-around time, for instance, would allow for rapid  
20 optimization of transgene expression and protein secretion. It would also provide a supply of protein adequate for product processing studies and preclinical trials.

The hen offers a unique system for efficient direct transgenesis of the magnum gland, but initial attempts have yielded poor results. Plasmid DNAs carrying transgenes have been introduced directly into the magnum of mature hens  
25 by electroporation (Ochiai et al., *Poultry Science*, 1998, 77:299-302). Due to the large size of the oviduct of mature hens, the transient persistence of the plasmid DNAs in the cells and the inefficiency of organ electroporation, only very low levels of protein were detected in the oviduct tissue of sacrificed hens and no expressed protein was reported as being detected in the egg. Other attempts have

involved the transfection of magnum cells with expression cassettes only after excision of the cells from the bird and preparation of an oviduct cell culture (Sanders et al., *Endocrinology*, 1985, **116**:398-405; Sanders et al., *Biochemistry*, 1988, **27**:6550-6557; Schweers et al., *Journal of Biological Chemistry*, 1990, **265**:7590-7595; Otten et al., *Molecular Endocrinology*, 1988, **2**:143-147; Schweers et al., *Journal of Biological Chemistry*, 1991, **266**:10490-10497).

Thus, there exists a need for a rapid route to the expression of an exogenous protein in an avian oviduct and the resulting deposition of the protein in the avian egg.

10

## SUMMARY OF THE INVENTION

The present invention provides methods for producing transgenic avians by direct oviduct transgenesis which satisfy the need for a rapid route to the expression of an exogenous protein in an avian oviduct and deposition of the expressed protein in the avian egg.

In one embodiment of the invention, a method for preparing a transgenic avian which expresses an exogenous protein substantially only in its oviduct is provided that comprises delivering a nucleic acid expression cassette directly to the oviduct of an immature avian, wherein the nucleic acid expression cassette comprises (i) a promoter which is active in the avian oviduct and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter.

In another embodiment of the invention, a method for preparing a transgenic avian which lays eggs containing exogenous protein is provided. The method of this embodiment comprises first preparing a transgenic avian which expresses an exogenous protein substantially only in its oviduct (as described in the previous paragraph) and then allowing the immature avian to grow to maturity,

so that the exogenous protein is secreted into the oviduct lumen of the mature avian and deposited into eggs laid by the avian.

In another embodiment of the invention, a method for preparing a transgenic avian which lays eggs containing exogenous protein is provided which  
5 comprises delivering a nucleic acid expression cassette directly to the oviduct of a mature avian, where the nucleic acid expression cassette comprises (i) a promoter which is active in the avian oviduct and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter, and where the exogenous protein is expressed in the avian's oviduct, secreted into the lumen  
10 of the oviduct, and deposited into eggs laid by the avian.

In an alternative embodiment of the invention, a method for producing an avian egg that contains exogenous protein is provided. The method of this embodiment comprises first delivering directly to the oviduct of an immature or mature avian a nucleic acid expression cassette which comprises (i) a promoter  
15 which is active in the avian oviduct and (ii) a nucleic acid sequence which is operably linked to the promoter and which codes for an exogenous protein, and then, if the avian is immature, allowing the avian to grow to maturity, wherein the exogenous protein is expressed in the oviduct, secreted into the lumen of the oviduct, and deposited into eggs laid by the avian.

20 Still another embodiment of the invention provides a method for producing a protein. This method comprises producing an avian egg that contains exogenous protein as previously described and then isolating the exogenous protein from the egg.

A method for testing the efficiency of the expression of a transgene in an  
25 avian oviduct is also provided which comprises delivering a nucleic acid expression cassette directly to the oviduct of an avian, wherein the expression cassette comprises (i) a promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter, and assaying for the presence or amount of the exogenous protein in the lumen of the oviduct.

In another embodiment of the invention, a method for testing the ability of a promoter to facilitate expression of a transgene in an avian oviduct is provided which comprises the following steps: delivering a nucleic acid expression cassette directly to the oviduct of an avian, where the nucleic acid expression cassette  
5 comprises (i) the promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter; and assaying the lumen fluid of the oviduct for the amount or presence of the exogenous.

In another embodiment, the invention provides a method for screening a preparation of retroviral particles for deleterious mutations. The method of this  
10 embodiment comprises first delivering the retroviral particles from a single preparation to the oviduct of an avian, wherein the retroviral particles contain nucleic acid expression cassettes comprising (i) a promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter. In the next step, the method comprises assaying for the presence or  
15 amount of the exogenous protein in the fluid of the lumen of the oviduct.

In an alternative embodiment of the invention, a method for testing the suitability of a transgene for expression in an avian oviduct or for secretion of its expression product into the lumen of the oviduct and into the eggs of an avian is provided which comprises the following steps: delivering a nucleic acid  
20 expression cassette directly to the oviduct of an immature avian, wherein the expression cassette comprises (i) a promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter; allowing the immature avian to grow to maturity; and assaying for the amount or presence of the exogenous protein in the egg white of eggs laid by the  
25 avian.

In further embodiments, the present invention provides for avians having a transgene encoding an exogenous protein substantially only in their oviducts, where the avians secrete into their eggs the protein expressed by the transgene. The invention also provides for avians having an exogenous protein substantially



only in their oviducts, where the avians secrete the exogenous protein into their eggs.

An intact avian egg containing protein exogenous to an avian egg is further provided by the present invention.

5 In still other embodiments of the invention, expression vectors and delivery vehicles particularly useful for the methods of the invention are provided. For instance, an avian leukosis virus pseudotyped with the G envelope glycoprotein of the vesicular stomatitis virus is provided. Additionally, an isolated polynucleotide comprising the sequence set forth in SEQ ID NO:1, the complement thereof, an at  
10 least 12 nucleotide-long fragment thereof, or a sequence that hybridizes thereto, wherein the polynucleotide is not a fragment of an interferon alpha-2b gene found in nature, is provided. Preferably, the fragments are at least about 18 nucleotides long. Most preferably, the fragments are at least about 60 nucleotides long. An expression vector comprising the polynucleotide sequence operably linked to a  
15 promoter is also provided.

## DETAILED DESCRIPTION OF THE INVENTION

### 20 a) Definitions and General Parameters

The following definitions are set forth to illustrate and define the meaning and scope of the various terms used to describe the invention herein.

The term "avian" means a bird of any known species or type. The term includes the various known strains of *Gallus gallus*, or chickens, (for example,  
25 White Leghorn, Brown Leghorn, Barred-Rock, Sussex, New Hampshire, Rhode Island, Australorp, Minorca, Amro, California Gray, Italian Partidge-colored), as well as turkeys, pheasants, quails, duck, and other poultry commonly bred in commercial quantities.

“Direct delivery” refers to delivery which specifically and physically targets one cell type, tissue, compartment, or organ and avoids even transient exposure of other sites of the organism.

A “nucleic acid sequence” or “polynucleotide” includes, but is not limited to, eucaryotic mRNA, cDNA, genomic DNA, and synthetic DNA and RNA sequences, comprising the natural nucleoside bases adenine, guanine, cytosine, thymidine, and uracil. The term also encompasses sequences having one or more modified nucleosides. The terms “polynucleotide” and “oligonucleotide” are used interchangeably herein. No limitation as to length or to synthetic origin are suggested by the use of either of these terms herein.

A “coding sequence” or “open reading frame” is a polynucleotide or nucleic acid sequence which is transcribed (in the case of DNA) or translated (in the case of mRNA) into a polypeptide *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5’ (amino) terminus and a translation stop codon at the 3’ (carboxy) terminus. A transcription termination sequence will usually be located 3’ to the coding sequence.

Nucleic acid “control sequences” refers to translational start and stop codons, promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, as necessary and sufficient for the transcription and translation of a given coding sequence in a defined host cell. Examples of control sequences suitable for eucaryotic cells are promoters, polyadenylation signals, and enhancers. All of these control sequences need not be present in a recombinant vector so long as those necessary and sufficient for the transcription and translation of the desired gene are present.

A “signal peptide” is responsible for transport of the protein out of the cell. In the majority of secreted proteins, the sequence is at the N-terminus of the nascent protein and is cleaved during synthesis and translocation into the

endoplasmic reticulum. Secretory DNA sequences in general are derived from genes encoding secreted proteins of the same species of the transgenic animal. Such secretory DNA sequences are preferably derived from genes encoding polypeptides secreted from the cell type targeted for tissue-specific expression.

5 Secretory DNA sequences, however, are not limited to such sequences.

“Operably or operatively linked” refers to the configuration of the coding and control sequences so as to perform the desired function. Thus, control sequences operably linked to a coding sequence are capable of effecting the expression of the coding sequence. A coding sequence is operably linked to or  
10 under the control of transcriptional regulatory regions in a cell when DNA polymerase will bind the promoter sequence and transcribe the coding sequence into mRNA that can be translated into the encoded protein. The control sequences need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet  
15 transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered “operably linked” to the coding sequence.

The term “exogenous protein” means a protein or polypeptide not naturally present in a particular tissue or cell, a protein that is the expression product of an  
20 exogenous gene, an exogenous expression construct or a transgene, or a protein not naturally present in a given quantity in a particular tissue or cell.

The terms “heterologous” and “exogenous” as they relate to nucleic acid sequences such as coding sequences and control sequences, denote sequences that are not normally associated with a region of a recombinant construct, and/or are  
25 not normally associated with a particular cell or tissue. Thus, a “heterologous” region of a nucleic acid construct is an identifiable segment of nucleic acid within or attached to another nucleic acid molecule that is not found in association with the other molecule in nature. For example, a heterologous region of a construct could include a coding sequence flanked by sequences not found in association

with the coding sequence in nature. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene).

Similarly, a host cell transformed with a construct which is not normally present in  
5 the host cell would be considered heterologous for purposes of this invention.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, naturally-occurring polynucleotides or polypeptides present in a living animal are not isolated, but the same polynucleotides or polypeptides could be part  
10 of a vector or composition, and be isolated in that such vector or composition is not part of its natural environment.

"Magnum" is that part of the oviduct between the infundibulum and the isthmus containing tubular gland cells that synthesize and secrete the egg white proteins of the egg.

15 A "magnum-specific" promoter is a promoter which is primarily or exclusively active in the tubular gland cells of the magnum.

The expression products described herein may consist of proteinaceous material having a defined chemical structure. However, the precise structure depends on a number of factors, particularly chemical modifications common to  
20 proteins. For example, since all proteins contain ionizable amino and carboxyl groups, the protein may be obtained in acidic or basic salt form, or in neutral form. The primary amino acid sequence may be derivatized using sugar molecules (glycosylation) or by other chemical derivatizations involving covalent or ionic attachment with, for example, lipids, phosphate, acetyl groups and the like, often  
25 occurring through association with saccharides. These modifications may occur *in vitro*, or *in vivo*, the latter being performed by a host cell through post-translational processing systems. Such modifications may increase or decrease the biological activity of the molecule, and such chemically modified molecules are also intended to come within the scope of the invention.

Alternative methods of cloning, amplification, expression, and purification will be apparent to the skilled artisan. Representative methods are disclosed in Sambrook, Fritsch, and Maniatis, *Molecular Cloning, a Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory (1989).

5       “Vector” means a polynucleotide comprised of single strand, double strand, circular, or supercoiled DNA or RNA. A vector typically comprises some combination of the following elements operatively linked at appropriate distances for allowing functional gene expression: replication origin, promoter, enhancer, 5’ mRNA leader sequence, ribosomal binding site, coding sequence, termination and  
10 polyadenylation sites, and selectable marker sequences. One or more of these elements may be omitted in specific applications.

In some embodiments the promoter will be modified by the addition or deletion of sequences, or replaced with alternative sequences, including natural and synthetic sequences as well as sequences which may be a combination of  
15 synthetic and natural sequences. Many eukaryotic promoters contain two types of recognition sequences: the TATA box and the upstream promoter elements. The former, located upstream of the transcription initiation site, is involved in directing RNA polymerase to initiate transcription at the correct site, while the latter appears to determine the rate of transcription and is upstream of the TATA box.  
20 Enhancer elements can also stimulate transcription from linked promoters, but many function exclusively in a particular cell type. Many enhancer/promoter elements derived from viruses, e.g. the SV40, the Rous sarcoma virus (RSV), and cytomegalovirus (CMV) promoters are active in a wide array of cell types. The nucleic acid sequence inserted in the cloning site may have any open reading  
25 frame encoding a polypeptide of interest, with the proviso that where the coding sequence encodes a polypeptide of interest, it should lack cryptic splice sites which can block production of appropriate mRNA molecules and/or produce aberrantly spliced or abnormal mRNA molecules.

The termination region which is employed primarily will be one of convenience, since termination regions appear to be relatively interchangeable. The termination region may be native to the intended nucleic acid sequence of interest, or may be derived from another source.

5       A vector is preferably constructed so that the particular coding sequence is located in the vector with the appropriate regulatory sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control sequences. Modification of the sequences encoding the particular protein of  
10 interest may be desirable to achieve this end. For example, in some cases it may be necessary to modify the sequence so that it may be attached to the control sequences with the appropriate orientation; or to maintain the reading frame. The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector. Alternatively, the coding sequence can  
15 be cloned directly into an expression cassette which already contains the control sequences and an appropriate restriction site which is in reading frame with and under regulatory control of the control sequences.

The terms "expression construct", "nucleic acid expression cassette" and "transgene" are used interchangeably herein. The terms refer to heterologous  
20 polynucleotide sequences containing a desired coding sequence and control sequences in operable linkage, so that cells transformed with these sequences are capable of producing the encoded product. In order to effect transformation, the expression cassette may optionally be included on a discrete vector; however, the relevant polynucleotide may also be an integrative vector which has or can  
25 become integrated into the host chromosome.

Suitable marker sequences for identification and isolation of correctly modified cells include green, yellow, and blue fluorescent proteins (GFP, YFP, and BFP, respectively). Other suitable markers include thymidine kinase (tk), dihydrofolate reductase (DHFR), and aminoglycoside phosphotransferase (APH).

The latter imparts resistance to the aminoglycoside antibiotics, such as kanamycin, neomycin, and geneticin. These, and other marker genes such as those encoding chloramphenicol acetyltransferase (CAT),  $\beta$ -lactamase ( $\beta$ -la), and  $\beta$ -galactosidase ( $\beta$ -gal), may be incorporated into the primary nucleic acid cassette along with the  
5 gene expressing the desired exogenous protein, or the selection markers may be contained on separate vectors and cotransfected.

A "reporter gene" is a gene that "reports" its activity in a cell by the presence of the protein that it encodes.

A "plasmid" is a small, circular DNA vector capable of independent  
10 replication within a bacterial or yeast host cell.

A "delivery vehicle" facilitates or helps facilitate the entry of a heterologous DNA or RNA sequence into a cell. The delivery vehicle may optionally be a virus which encapsulates a vector containing the expression cassette and aids its transfer into the cell. In some cases, the viral delivery vehicle  
15 even integrates the transgene it carries into the genome of the host cell it infects. Alternatively, the delivery vehicle may be non-viral, such as a liposome.

A "regulatory element" is a DNA sequence contained within a gene to which a transcription factor(s) binds and alters the activity of the gene's promoter either positively (induction) or negatively (repression).

20 The terms "transformation", "transduction", and "transfection" all denote the introduction of a polynucleotide into a cell, such as an avian tubular gland cell.

The term "sequences which hybridize thereto" means polynucleotide sequences which are capable of forming Watson-Crick hydrogen bonds with another polynucleotide sequence under normal hybridization conditions, such as in  
25 buffered (pH. 7.0-7.5) aqueous, saline solutions (for instance, 1 to 500 mM NaCl) at room temperature. Although normal hybridization conditions will depend on the length of the polynucleotides involved, typically they include the presence of at least one cation such as  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ , or  $\text{Ca}^{2+}$ , a near neutral pH, and temperatures less than 55°C. Although the sequences which hybridize to a

polynucleotide may be about 90%-100% complementary to the polynucleotide, if the sequences are of sufficient length, in solutions with high salt concentrations, and/or under low temperature conditions, polynucleotides with complementarity of 70% or above, or even just 50% or above, may hybridize to the polynucleotide.

- 5 Sequences which hybridize thereto typically comprise at least 12 nucleotides, and preferably at least about 15 nucleotides, which are complementary to the target polynucleotide.

b) Direct Oviduct Transgenesis

- 10 The present invention is directed to methods of producing transgenic birds and producing exogenous proteins which are deposited in the eggs of birds, each of which involves the delivery of an expression cassette directly to the oviduct of an avian, such as a chicken.

- One embodiment of the invention provides a method for preparing a  
15 transgenic avian which expresses an exogenous protein substantially only in its oviduct. This method comprises delivering a nucleic acid expression cassette directly to the oviduct of an immature avian, wherein the nucleic acid expression cassette comprises (i) a promoter which is active in the avian oviduct and (ii) a nucleic acid sequence which codes for an exogenous protein and which is  
20 operably linked to the promoter.

- Preferably, the avian of the method described in the previous paragraph is allowed to grow to maturity. The exogenous protein which is expressed in the oviduct is secreted into the oviduct lumen of the avian and is deposited into eggs laid by the avian. Thus, in another embodiment of the invention, a method for  
25 preparing a transgenic avian which lays eggs containing exogenous protein is provided which comprises first preparing a transgenic avian which expresses an exogenous protein substantially only in its oviduct as described in the previous paragraph and then allowing the immature avian to grow to maturity.



In an alternative embodiment of the invention, a method of producing an avian egg which contains exogenous protein is provided. This method comprises delivering an expression cassette directly to the oviduct of an immature avian, where the expression cassette comprises the operably linked pair of (i) a promoter  
5 which is active in the avian oviduct and (ii) a nucleic acid sequence which codes for an exogenous protein. This method requires the additional step of allowing the immature avian to mature. When the avian reaches the stage where it begins to lay eggs (maturity), the exogenous protein which is expressed in the avian oviduct and secreted into the lumen of the oviduct is deposited into the eggs laid by the  
10 avian.

In another embodiment of the invention, a method for producing protein is provided which comprises first producing an avian egg that contains exogenous protein as described in the previous paragraph and then isolating the exogenous protein from the avian egg.

15 In all of the methods of the invention, the nucleic acid expression cassette is preferably delivered directly to the magnum cells of the immature oviduct.

The avian in each of the methods of the invention may optionally be selected from the group consisting of a chicken, turkey, goose, and quail.

In a preferred embodiment of each of the methods of the invention, the  
20 immature avian is an immature chicken. An immature chicken is a chicken at a stage between hatch and the time when she begins to lay eggs (about 20 to about 24 weeks old). In a further preferred embodiment, the immature chicken is from about 10 to about 20 weeks old. At 10-12 weeks of age, the pullet oviduct weighs only 0.2 grams and the magnum consists of about  $4 \times 10^7$  cells. By 20 weeks,  
25 which is when most hens begin to lay eggs, the oviduct has matured into a 30-50 gram organ with a parallel increase in tubular gland cell number. By accessing the oviduct for transgenesis before it is mature, a higher percentage of cells can be modified, as compared to a mature hen oviduct.

In preferred embodiments of the methods of the invention, the immature avian is subjected to a steroid hormone treatment prior to delivery of the expression cassette. The steroid treatment comprises the administration of estrogen, progesterone, or testosterone, or combinations thereof. From hatch to 17 weeks, the cells of the magnum of chickens can be transiently induced to undergo a burst of proliferation by combined treatment with estrogen, progesterone, and/or testosterone (Yu & Marquardt, *Poultry Science*, 1973, 53:1096-1105; Brant & Nalbandov, *Poultry Science*, 1956, 35:692-700; Kohler et al., *J. Cell. Biol.*, 1969, 40:8-27; Oka and Schimke, *J. Cell Biol.*, 1969, 43:123-137; Palmiter and Wrenn, *J. Cell Biol.*, 1971, 50:598-615). Cessation of hormone treatment allows the magnum cells to resume their normal developmental program. In the present invention, the steroid treatment preferably comprises administration of estrogen, either alone or, preferably, in combination with progesterone or testosterone.

Estrogen alone can optionally be used alone either in the form of an implanted 13-15 mg diethylstilbestrol pellet or by injections of estrogen dissolved in a carrier. Injections of the hormone are preferred to implantation of a pellet because cessation of hormonal treatment is easier and two or more different hormones can be coinjected. Estrogen can be in the form of estrone, estradiol or diethylstilbestrol (DES); but DES is preferred because it is the most potent form. Doses can range from 0.1 mg to 24 mg per bird, depending on the weight of the bird. Typically doses for a 1 kg hen will be 1 mg of DES injected intramuscularly (typically the breast muscle), once per day, in a volume of 0.1 ml of 95% ethanol, Mazola oil or sesame oil.

While estrogen alone can induce proliferation of immature magnum cells, cotreatment with progesterone or testosterone greatly enhances the proliferative effect of estrogen. Therefore DES is preferably coinjected either with progesterone or testosterone, and most preferably with progesterone because testosterone is difficult to obtain due to its classification by the Drug Enforcement Agency as an anabolic steroid. For a 1 kg pullet (a young domestic hen), 1 to 3

mg of testosterone or 0.1 to 0.8 mg of progesterone should be coinjected along with 1.0 mg of DES. The preferred method is to dissolve 10 milligrams (mg) of DES and 8 mg of progesterone per milliliter (ml) of sesame oil. 0.1 ml doses are applied once a day by injection into the breast muscle.

5 In an alternative embodiment of the invention, an immature, non-chicken avian is subjected to a steroid hormone treatment prior to the delivery of the expression cassette. Estrogen and progesterone have been shown to have effects on the immature oviduct of Japanese Quails, ducks, and gulls that are similar to those in chicken (see Korpela et al., *Gen. Comp. Endocrinol.*, 1981, 44: 230-232).

10 One of ordinary skill in the art will be able to readily adapt dosage recommendations established for chickens to other types of birds.

For an avian of any type which is subjected to the methods of the invention, the hormone treatment is preferably administered daily and is optionally begun from about two to about ten days before introduction of the expression cassette  
15 into the magnum. Either directly after transfection or transduction of the magnum or up to about 1 to 2 days later, the hormone administration is ceased, allowing the oviduct to develop normally.

In a preferred embodiment, the hormones are injected in the morning on days 1 through 3 (see dosages listed above). A procedure for directly accessing  
20 the oviduct such as surgery or laparoscopy, including injection of the expression vector into the lumen of the magnum, is performed in the morning of day 4. Additional hormone injections are performed in the afternoon of day 4 and in the morning of day 5. The day 5 injection is optional. Depending on when the procedure is carried out, the treated hen will start to lay eggs containing the  
25 desired protein within four to ten weeks.

Alternatively, all of the methods of the invention may comprise delivery of the nucleic acid expression cassette directly to the oviduct of a mature avian (an avian which is already laying eggs), instead of the preferred immature avian. For instance, an alternative method for preparing a transgenic avian which lays eggs

containing exogenous protein comprises delivering a nucleic acid expression cassette directly to the oviduct of a mature avian, wherein the nucleic acid expression cassette comprises (i) a promoter which is active in the avian oviduct and (ii) a nucleic acid sequence which codes for an exogenous protein and which  
5 is operably linked to the promoter, where the exogenous protein is expressed in the avian's oviduct, secreted into the lumen of the oviduct and deposited into eggs laid by the avian.

As a further example of how the methods of the invention may be adapted for use with mature avians, an alternative method for producing an avian egg that  
10 contains exogenous protein, comprises delivering a nucleic acid expression cassette directly to the oviduct of an immature avian, wherein the nucleic acid expression cassette comprises (i) a promoter which is active in the avian oviduct and (ii) a nucleic acid sequence which is operably linked to the promoter and which codes for an exogenous protein, where the exogenous protein is expressed  
15 in the oviduct, secreted into the lumen of the oviduct, and deposited into eggs laid by the avian.

In any of the methods of the invention, application of the expression cassette directly to the oviduct can be achieved by surgery. A surgical procedure involving an opening of the abdominal wall, is one option.

20 In a preferred embodiment, the surgical procedure used to access the avian oviduct comprises endoscopy of the abdominal cavity or laparoscopy. The laparoscope is used to visualize the oviduct while a fine gauge needle is inserted via a cannula and solution containing the expression cassette (optionally in a delivery vehicle such as a virus) is injected into the lumen of the magnum.  
25 Laparoscopic grasping forceps or a suction clamp hooked to a vacuum source can be used to secure the oviduct.

In an alternative embodiment, a non-surgical method of directly accessing the oviduct for transgenesis may be used instead. One such suitable non-surgical method is transcloacal catheterization. Transcloacal catheterization comprises

first inserting a needle or tube (preferably a feeding needle) into the cloaca. The needle is then fed through the shell gland, isthmus, and into the lumen of the magnum. The solution is injected when the feeding needle reaches this position. The whole catheterization process may optionally be image-guided.

- 5           In an alternative embodiment of the invention, a method for preparing a transgenic avian which expresses an exogenous protein substantially only in its oviduct is provided which comprises delivering a nucleic acid expression cassette directly to the oviduct of an avian via laparoscopy or transcloacal catheterization, wherein the nucleic acid expression cassette comprises (i) a promoter which is  
10   active in the avian oviduct and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter.

- The expression cassette which is used in the methods of the invention must comprise the coding sequence of the exogenous protein of interest. The expression cassette must also contain a promoter which is active in cells of the  
15   magnum and is operably linked with the nucleic acid sequence encoding the exogenous protein to be expressed. In one embodiment of the invention, a constitutive promoter, such as a cytomegalovirus (CMV), Rous sarcoma virus (RSV), or  $\beta$ -actin promoter is used. It is a distinct advantage of this method over traditional transgenic approaches that this method does not require tissue-specific  
20   promoter elements. Alternatively, a magnum-specific promoter, such as the ovalbumin, conalbumin, ovomucoid, transferrin, or lysozyme promoter or a portion thereof, can instead be used in the methods of the present invention.

- The exogenous protein which is expressed in the avian oviduct preferably comprises a signal peptide sequence (encoded by the nucleic acid expression  
25   cassette delivered to the avian oviduct) which facilitates secretion of the protein into the lumen of the oviduct and deposition of the protein into eggs laid by the avian. The protein which is expressed in the oviduct may be optionally expressed with its own native signal peptide that allows it to be efficiently secreted into the lumen of the oviduct and deposited into an egg. However, in other embodiments

the expression cassette may further comprise an operably linked sequence which codes for a signal peptide that is heterologous to the exogenous protein but which can still direct secretion of the exogenous protein into the lumen of the oviduct. A polynucleotide coding for an engineered signal peptide such as that occurring in egg white prelysozyme may be included in the expression cassette and operably linked to the coding sequence of the expression cassette (Jigami et al., *Gene*, 1986, 43:273-279). Such a signal peptide would preferably be expressed as a fusion protein with the exogenous protein of interest. The presence of the signal peptide allows secretion of the protein into the lumen of the magnum, and subsequently into the egg.

For purposes of application to the oviduct, the expression cassette is typically inserted within a larger vector to create an expression vector. The vector may optionally be an autonomously replicating vector. Alternatively, the vector may optionally be an integrative vector. In such an embodiment, the expression cassette is flanked by integration sequences. In addition to the coding sequence, the promoter and the optional secretory DNA sequence, the expression cassette or vector may also include assorted additional regulatory elements and control sequences such as enhancers. For instance, the SV40 enhancer is known to be useful in increasing expression from the ovalbumin promoter. Termination signals, splicing signals, and the like may also be included on the expression vector. A reporter or marker sequence may optionally be included on the expression vector as well.

Transfection of the magnum cells with plasmid DNAs containing the expression cassette can be performed using a chemical-mediated method or a ballistic-type delivery system, such as a gene-gun. Transfection of plasmid DNAs normally results in transient expression in the magnum as the plasmids are lost over time. Alternative embodiments offer the means of obtaining persistence of the plasmid's transgene within the oviduct. In one of these embodiments, a DNA replication sequence is included on the plasmid so that it will replicate stably and

persistently during magnum cell proliferation and differentiation. In another embodiment, the transgene is flanked with integration sequences and cotransfected with a plasmid encoding protein(s) capable of integrating the transgene into the chicken genome.

5            Preferably, the delivery of the expression cassette to the cells of the oviduct is aided by the use of a delivery vehicle. In a preferred embodiment of the present invention, the expression cassette is delivered directly to the oviduct within a delivery vehicle such as an adenovirus, a retrovirus, or a liposome. Alternatively, other types of viruses may be used as delivery vehicles.

10            To introduce transgenes directly into magnum cells, a variety of viral-mediated methods can be used. Human adenoviruses, such as those described in Fisher and Watanabe, *Cardiovascular Research*, 1996, 31:E86-95 and Lou et al., *Journal of Orthopedic Research*, 1996, 14:513-517, both herein incorporated by reference, have been shown to be efficient means of introducing genes into  
15            chicken cells. Very high titers can be achieved ( $10^{10}$ ), thus allowing a high multiplicity of infection (MOI). The human adenovirus vectors described above are replication-deficient and do not integrate into magnum cell DNA. Thus, the transgene persists only transiently in cells and long-term expression is not expected. Therefore, when a replication-defective adenovirus is used as a delivery  
20            vehicle it is preferable to introduce the adenovirus vector into the magnum just before or during the onset of lay (20 weeks of age).

                 In another embodiment of the invention which can offer long-term expression, any retrovirus able to infect magnum cells may be used to introduce the transgene into magnum cells. Because retroviruses require rapidly dividing  
25            cells to integrate efficiently into the host cell's DNA, use of the optional steroid hormone treatment on the immature avian prior to delivery of the nucleic acid cassette will help promote efficient transgenesis of the magnum cells. Therefore, when a retrovirus is used as a delivery vehicle for the expression cassette, prior

administration of the steroid hormone treatment to the immature chicken is strongly preferred.

For example, in preferred embodiments of the invention, a replication-deficient retrovirus derived from the avian leukosis virus (ALV) is used in the methods of the invention as a delivery vehicle for the expression cassette. An ALV-based retroviral vector has been used to produce germline transgenic Brown Leghorns (Cosset et al., *Journal of Virology*, 1991, 65:3388-94; Thoraval et al., *Transgenic Research*, 1995, 4:369-377). High titers can be achieved ( $5 \times 10^6$ ), but this virus requires that the target cell be dividing. The target magnum cells of an immature bird are undergoing rapid mitosis, especially after treatment of the bird with hormones. The ALV retroviral vector is replication-deficient. Thus, upon integration of the transgene into the chicken's genome, no virus will be produced during subsequent maturation of the oviduct and the egg laying lifetime of the hen.

In another embodiment of the methods of the invention, the vesicular stomatitis virus (VSV) pantropic retrovirus is used to deliver the expression cassette. The VSV pantropic vector, LZRN-L-G encodes  $\beta$ -galactosidase under control of the Moloney murine leukemia virus and is packaged with the G envelope glycoprotein (G-protein) of the vesicular stomatitis virus (VSV-G) (see Burns et al., *Proc. Natl. Acad. Sci. USA.*, 1993, 90:8033-8037). This transducing vector attaches directly to the outer phospholipid layer of cells in a receptor-independent manner. Hence, this transducing particle has been shown to integrate into the genomes of a variety of species, including chickens (Burns et al., *Proc. Natl. Acad. Sci. USA.*, 1993, 90:8033-8037; Lin et al., *Science*, 1994, 265:666-669; Yee et al., *Methods in Cell Biology*, 1994, 43:99-112). The VSV pantropic vector is replication-deficient. Thus, upon integration of the transgene into the chicken's genome, no virus will be produced during subsequent maturation of the oviduct and the egg laying lifetime of the hen.



The primary advantage of LZARNL is the G-protein, which allows the vector particles to adsorb to tissues in a receptor-independent manner. This allows LZARNL to transduce a wide range of tissues. However, LZARNL is not an ideal vector for insertion of magnum expression cassettes due to poor cloning sites and limited room between the long terminal repeats (LTRs). The ALV-based vector, NLB, overcomes these deficiencies. However NLB is pseudotyped with an envelope protein that requires it to adsorb to cells via the cell-surface TV receptor. There are several ALV envelope proteins, each of which interacts with a specific TV receptor. For instance the envA envelope protein adsorbs to the TV-A receptor. The TV-A receptors, as well as other TV receptors are expressed on an unknown percentage of avian cells. For instance, almost all chicken fibroblast cells but only a small percentage of stage X embryo cells express the TV-A receptor. It is unknown whether chicken magnum cells express the TV-A receptor at levels adequate for transduction by ALV particles pseudotyped with the envA envelope protein. Therefore ALV particles alone may not be able to enter into magnum cells.

One aspect of the present invention provides for an ALV particle in which the G protein is inserted into the envelope of the ALV particle. This allows production of NLB vector packaged in a particle able to transduce cells directly through the phospholipid membrane, bypassing the requirement for cell-surface TV receptors. As an example of how an ALV-based retrovirus may be pseudotyped with the G envelope glycoprotein of the vesicular stomatitis virus, the cells which package the NLB vector encoding a CMV promoted interferon (IFN) gene are Isalde cells (which produce the gag, pol and envA proteins and are referred to as Isalde-NLB-CMV-IFN). The particles produced by this cell line are pseudotyped with the envA receptor, which specifically adsorbs to the TV-A receptor. Isalde-NLB-CMV-IFN cells are plated out at  $3-5 \times 10^6$  cells per T75 tissue culture flask. The next day, they are transfected with an expression plasmid that expresses the G protein under control of the CMV promoter (pCMV-G) using

standard transfection protocols. In the following days, the transfected cells will produce NLB-CMV-IFN particles, of which a significant fraction are now pseudotyped with the G-protein. See the Example 3, below for a working example of a pseudotyping protocol. ALV particles pseudotyped with the G envelope glycoprotein have been found to successfully transduce the magnum cells of the chicken oviduct (see Example 4, below), whereas attempts to transduce magnum cells with ALV particles pseudotyped with the envA protein have not been successful.

It will be apparent to one of ordinary skill in the art that retroviruses other than the ones specifically mentioned here will be useful in the direct oviduct transgenesis methods described herein. Both replication-defective and replication-competent retroviruses can be used for the present invention, although replication-defective retroviruses are preferred.

Alternatively, instead of directly injecting a virus that comprises the expression cassette into the magnum in the methods of the invention, one injects the cells which produce the virus (helper cells). The helper cells can live for several days inside the magnum. During that time, the helper cells continuously secrete virus, thereby increasing the dosage and duration of the infection of the magnum. Any helper cell known to those skilled in the art to be compatible with the viral delivery vehicle chosen for use in the methods of the invention would be suitable for this application.

For instance, in one particular embodiment, a helper cell line that is producing high titers of ALV-based retrovirus is transfected with a vector encoding the VSV-G protein. The following day the helper cells will secrete ALV-based retroviruses containing the VSV-G protein inserted into the envelope. The cells are harvested in a manner that maintains viability and are typically immediately injected into the magnum's lumen.

Liposomes provide still another option for a delivery vehicle useful in the methods of the present invention. Liposomes increase intracellular stability and

increase uptake efficiency. Liposomes are hollow spherical vesicles composed of lipids arranged in a similar fashion as those lipids which make up the cell membrane. The appropriate use of liposomes as delivery vehicles in the present invention would be known to one of ordinary skill in the art. Numerous lipids are commercially available, and some, such as Lipofectin™, have been used to deliver genes into a variety of organs.

The delivery of the expression vector directly to the magnum portion of the oviduct can optionally be further aided by positioning an expression-cassette releasing entity directly in or adjacent to the magnum. The expression-cassette releasing entity can be a packet or suppository which releases plasmid DNA, liposomes, or viruses carrying the expression cassette into the magnum over some period of time. Alternatively, as discussed above with respect to retroviruses, the deposited expression-cassette releasing entity is a helper cell which both produces and secretes viral delivery vehicles into the oviduct.

As previously stated, the magnum can be treated at almost any time during development but certain times are particularly advantageous. At 10 to 15 weeks, the magnum of an avian such as a chicken is small and more readily saturated by virus or another carrier of an expression cassette. If retroviral delivery is used, direct oviduct transgenesis at this early stage may be preferred. In the case of retrovirus treatment, primordial magnum cells that contain the integrated transgene will amplify the transgene copy number (relative to the magnum) as the cells proliferate. After 15 weeks, the magnum of an avian such as a chicken is larger, requiring larger amounts of virus to facilitate efficient transgenesis. However, for transient transgenesis with adenovirus infection, liposome-mediated transfection, or plasmid-transfection, 15 to 20 weeks should be the optimal time period for treatment.

If a mature magnum is to be targeted, instead of the preferred immature magnum, then non-retroviral delivery methods such as liposome delivery are most suitable.

If a retrovirus is used as a delivery vehicle, it is especially advantageous to administer the aforementioned steroid hormone treatment to the immature avian prior to infection with the virus due to the resulting stimulation in growth and DNA synthesis. In other embodiments of the invention, however, alternative  
5 methods of stimulating magnum cell growth and DNA synthesis may be employed either alone or in conjunction with the aforementioned steroid hormone treatment. These modifications are likely to increase the integration efficiency of the retroviral vector used. One such alternative method involves the inclusion of an oncogene or growth factor gene in the genome of the retroviral vector.

10 Alternatively, when the magnum cells are infected with the retrovirus carrying the expression cassette, they may be coinfectd with a second virus which expresses an oncogene or growth factor. This second virus could be an adenovirus, which would offer the advantage of only transiently stimulating DNA synthesis, thereby not causing any detrimental effects to the bird.

15 Oncogenes/growth factors useful in the present invention include src, large T antigen of polyoma/SV-40 virus, E2F1, avian c-erb B, c-myc, and c-jun. These factors may optionally be coexpressed with an inhibitor of apoptosis (programmed cell death) such as bcl-2 or baculovirus p35. Over-expression of many oncogenes and growth factors can result in apoptosis, which is readily offset by expression of  
20 an apoptosis inhibitor such as bcl-2 or p35.

Use of the invention thus allows for targeted expression of exogenous proteins in oviduct cells with secretion into the lumen of the oviduct magnum and, ultimately, deposition in eggs whites. The exogenous protein may be packaged in the eggs in quantities of up to one gram or more per egg. The invention can be  
25 used to express, in large yields and at low cost, a wide range of desired proteins including those used as human and animal pharmaceuticals, diagnostics, and livestock feed additives. In preferred embodiments of the invention, the proteins which are expressed in the avian oviducts are human proteins. Proteins such as human growth hormone, interferon (such as interferon  $\alpha, \beta, \gamma$ , or  $\omega$ ), lysozyme, and

$\beta$ -casein are examples of proteins which are desirably expressed in the oviduct and deposited in eggs according to the methods of the invention. Other possible proteins to be expressed (or overexpressed) in the avian oviduct and isolated from eggs according to the methods of the invention include, but are not limited to,

5 albumin,  $\alpha$ -1 antitrypsin, antithrombin III, collagen, factors VIII, IX, X (and the like), fibrinogen, hyaluronic acid, insulin, lactoferrin, protein C, erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), tissue-type plasminogen activator (tPA), feed additive enzymes, somatotropin, and chymotrypsin. Genetically engineered

10 antibodies, such as immunotoxins which bind to surface antigens on human tumor cells and destroy them, can also be expressed for use as pharmaceuticals or diagnostics. In one preferred embodiment, the expression cassettes used in the methods of the invention code for human interferon alpha-2b.

Proteins which are isolated from avian eggs produced by the methods of the

15 invention are provided by additional aspects of the invention. For instance, an interferon, such as human interferon alpha-2b, which is isolated from eggs produced according to the methods of the invention is provided by the present invention.

In preferred embodiments of the methods of the invention, the sequence on

20 the expression cassette which codes for an exogenous protein not normally expressed in an avian, or at least not in an avian oviduct, has been modified to use codons which are commonly used in genes expressed by the avian and particularly genes expressed in the avian oviduct. One such coding sequence useful for expression in the chicken oviduct is the sequence shown in SEQ ID NO:1. This

25 sequence codes for pre-human interferon alpha-2b, but contains a number of altered codons to more closely match the codon usage preferences demonstrated by genes normally expressed in the chicken oviduct. Thus, an alternative aspect of the invention provides an isolated polynucleotide comprising the sequence set forth in SEQ ID NO:1, the complement thereof, an at least 12 nucleotide-long

fragment thereof, or a sequence that hybridizes thereto, wherein the polynucleotide is not a fragment of an interferon alpha-2b gene found in nature. Optionally, the signal peptide of the pre-human interferon alpha-2b coding sequence may be replaced with a heterologous signal peptide, such as that from lysozyme, for use in any of the methods of the invention. Expression vectors comprising this polynucleotide and a promoter which is operably linked to the polynucleotide are also provided by the present invention.

In another embodiment, the invention provides an avian having a transgene encoding an exogenous protein substantially only in its oviduct, where the avian secretes the exogenous protein into its eggs. In another embodiment, the invention provides an avian having an exogenous protein substantially only in its oviduct, where the avian secretes the exogenous protein into its eggs. Furthermore, the invention provides an avian which lays eggs containing exogenous proteins such as human proteins. Optionally, the exogenous protein which the avian secretes into its egg comprises a human interferon.

In still another embodiment, the present invention provides an intact avian egg that contains protein exogenous to the avian egg. Optionally, the exogenous protein contained in the egg comprises a human interferon, such as human interferons alpha-2b.

To demonstrate the potential of direct oviduct transgenesis (DOT), hormone-induced magnums of pullets were transduced with retroviral particles carrying the human interferon alpha-2b gene (IFN) under control of the CMV promoter (see the specific examples, Examples 1-4, below). The construction of the vector used for these experiments is outlined in the specific example, Example 1, below. The production of the retroviral cell lines and particles are detailed in the specific examples, Examples 2 and 3, below. The retroviral particles used for the working example of direct oviduct transgenesis were pseudotyped with VSV-G. One week after the DOT operation, lumen fluid was sampled from two hens (Example 4, below) and compared to supernatants from excised oviducts as

negative and positive controls (Example 5, below). Both DOT-treated hens had significant levels of human interferon in the lumen fluid (Example 4, Table 1, below). Eggs from the same birds were collected and assayed for the presence of interferon in the egg white (Example 4, Table 2, below).

5           In addition to the usefulness of direct oviduct transgenesis for preparing transgenic avians, expressing transgenes in the avian oviduct, preparing eggs containing exogenous protein, and producing proteins, direct oviduct transgenesis provides a quick route to assessing the usefulness of a particular genetic construct or part of a genetic construct for expression in the avian oviduct. For instance, the  
10   efficiency of promoters, such as magnum-specific promoters, or other regulatory elements may be rapidly tested. Alternatively, the coding sequence itself may be tested. For instance, the effectiveness of implementing codon changes within the coding sequence for expression in the magnum may be tested. Signal sequence effectiveness may also be tested using the methods of the invention. The tested  
15   expression constructs, if satisfactory, can then optionally be used to generate germline transgenic avians where expression in the oviduct is desired. For instance, the tested expression cassettes may be delivered via a retrovirus to a developing avian embryo in an egg (see co-pending US patent application no. 09/173,864, filed October 16, 1998, and Bosselman et al., US Patent No. 5,162,  
20   215).

          Although the following methods, like all the methods of the invention, are preferably carried out using an immature avian, each of the methods can alternatively be performed using a mature avian. (If a mature avian is used, one of ordinary skill in the art will recognize that any steps directed to allowing the bird  
25   to grow to maturity are no longer necessary.)

          One embodiment of the invention provides a method for testing the efficiency of the expression of a transgene in an avian oviduct which comprises first delivering a nucleic acid expression cassette directly to the oviduct of an immature avian, wherein the nucleic acid expression cassette comprises (i) a

promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter, and then assaying for the presence or amount of the exogenous protein in the lumen of the oviduct. In a preferred embodiment, the nucleic acid expression cassette is in a retroviral vector.

5 In another embodiment of the invention, a method for testing the ability of a promoter to facilitate expression of a transgene in an avian oviduct is provided which comprises the following steps: delivering a nucleic acid expression cassette directly to the oviduct of an immature avian, wherein the nucleic acid expression cassette comprises (i) the promoter and (ii) a nucleic acid sequence which codes  
10 for an exogenous protein and which is operably linked to the promoter; and assaying for the presence or amount of the exogenous protein in the fluid of the lumen of the oviduct. In a preferred embodiment, the promoter is a magnum-specific promoter.

Typically, transgenes can be quickly tested for expression in the magnum  
15 by sampling lumen fluid after the operation or other delivery of the nucleic acid expression cassette to the oviduct. Preferably, the lumen fluid is sampled at least about one week after delivery of the nucleic acid expression cassette to the oviduct and before the avian begins to lay eggs. Most preferably, the lumen fluid is sampled within about three to about six weeks after the operation (or other form of  
20 delivery of the nucleic acid expression cassette). An example of such a procedure is illustrated in the specific example, Example 4, below.

The method by which the sample of lumen fluid is assayed will be dependent upon the identity of the exogenous protein. One of ordinary skill in the art will readily be able to identify a suitable method of establishing the presence  
25 and/or amount of the exogenous protein within the fluid sample. In many instances, standard assays using antibodies or labeled substrates may be used.

The methods of the invention are especially useful for testing magnum-specific promoters in live birds. If the promoter of the expression cassette is magnum-specific, a hormone treatment is preferably administered to the immature



avian for at least about six to ten days after delivery of the nucleic acid expression cassette. If the preferred hormone treatment has been applied to the immature avian as previously described prior to delivery of the nucleic acid expression cassette to the oviduct, then the hormone treatment is preferably extended for  
5 about 6 to about 10 days past the operation to ensure that the magnum is capable of expressing the magnum-specific promoters. Significant expression of magnum-specific genes is not detected in pullets unless hormone treatment is continued for at least 10 days (Brant and Nalbandov, 1956; O' Malley et al., 1979, *Recent Prog. Horm. Res.*, 35:1-46; Oka and Schimke, 1969; Yu and Marquardt, 1973, *Comp.*  
10 *Biochem. Physiol. [B]*, 44:769-77; Yu and Marquardt, 1973, *Endocrinology*, 92:563-72). At about six to about ten days post-operation, the lumen fluid may be sampled and assayed for the presence of the desired secreted protein. In this way, transgenes with a magnum-specific promoter can be tested for activity.

A particular advantage of the methods of the invention is that they allow  
15 the rapid testing of magnum-specific promoters in retroviral expression vectors. Currently it is not possible to transduce magnum cells with retroviral vectors once they have been excised from the pullet or hen and cultured via the oviduct explant assay (OEA) (Sanders and McKnight, 1985, *Endocrinology*, 116:398-405; Sanders and McKnight, 1988, *Biochemistry*, 27:6550-7). Although it is unknown  
20 what is the block to transduction in the oviduct explant assay, it may be that the cells in the oviduct explant assay cease to divide and efficient transduction requires concomitant cellular proliferation. With direct oviduct transgenesis, the functionality of a magnum-specific promoter (from the *ovalbumin*, *lysozyme*, *conalbumin*, *ovomucoid* or *transferrin* genes, for example) in a preparation of  
25 transduction particles could be quickly confirmed. This would allow the screening of particle preparations for deleterious mutations that arose during the production of the particles. Particle preparations that demonstrate activity in the magnum after direct oviduct transgenesis would then be used to produce a flock of transgenic chickens.

Similarly, a method for testing the suitability of a transgene for expression in an avian oviduct and/or for secretion of its expression product into the lumen of the oviduct and into the eggs of an avian is also provided which comprises the following steps: delivering a nucleic acid expression cassette directly to the  
5 oviduct of an immature avian, wherein the nucleic acid expression cassette comprises (i) a promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter; allowing the immature avian to grow to maturity; and assaying for the amount or presence of the exogenous protein in the egg white of eggs laid by the avian. The method may  
10 optionally further comprise the additional step of assaying the exogenous protein found in the lumen or in the egg for bioactivity. Preferably, but not necessarily, the protein in the egg is assayed after isolation from the egg.

Direct oviduct transgenesis can also be used to confirm that a given protein can be expressed in the magnum of live pullets and secreted into the egg white of  
15 laying hens. A protein which is produced in this manner can then optionally be assayed to confirm bioactivity. Also, if the protein is present in the eggs in reasonably large quantities, the protein may be isolated and used for additional studies such as protein purification trials or efficacy studies.

In another embodiment of the invention, a method for screening a  
20 preparation of viral particles for deleterious mutations is provided which comprises the following steps: delivering the viral particles from a single preparation to the oviduct of an immature avian, wherein the viral particles contain nucleic acid expression cassettes comprising (i) a promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is  
25 operably linked to the promoter; and assaying fluid of the lumen of the oviduct for the amount of the exogenous protein which has been both expressed in the oviduct and secreted into the lumen of the oviduct. In a preferred embodiment, the viral particles comprise retroviral particles. In another preferred embodiment, the promoter of the viral particles' expression cassette is a magnum-specific promoter.

In another embodiment, the present invention provides a method for investigating the nature of an exogenous protein which has been expressed in an avian's oviduct and secreted into the egg white of the avian's eggs. The method of this embodiment comprises the following steps: delivering a nucleic acid  
5 expression cassette directly to the oviduct of an immature avian, wherein the nucleic acid expression cassette comprises (i) a promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter; allowing the immature avian to grow to maturity; and assaying the exogenous protein found in the eggs laid by the avian for a particular  
10 characteristic. In a preferred embodiment, the method further comprises the additional step of isolating the exogenous protein from the eggs laid by the immature avian prior to assaying the exogenous protein for the particular characteristic. The preferred characteristic may optionally be bioactivity or a structural characteristic. For instance, the nature of post-translational  
15 modifications on the exogenous protein which has been expressed in the avian oviduct may be investigated.

c) Examples

The following specific examples are intended to illustrate the invention and  
20 should not be construed as limiting the scope of the claims:

Example 1. Vector Construction.

To allow for the efficient replacement of the *lacZ* gene of pNLB with any transgene, an intermediate adaptor plasmid was first created, pNLB-Adapter.  
25 pNLB-Adapter was created by inserting the chewed back *ApaI/ApaI* fragment of pNLB (Cosset et al., *J. Virol.*, 1991, **65**:3388-94) into the chewed-back *KpnI/SacI* sites of pBluescriptKS(-). (In pNLB, the 5' *ApaI* resides 289 bp upstream of *lacZ*, and the 3' *ApaI* resides 3' of the 3' LTR and Gag segments). The filled-in *ClaI/XbaI* fragment of pCMV-IFN-MM (Moore et al., *Anal. Biochem.*, 1997,

247:203-209) was inserted into the chewed-back *KpnI/NdeI* sites of pNLB-Adapter, replacing *lacZ* with the CMV promoter and the *IFN-MM* gene and thereby creating pNLB-Adapter-CMV-IFN-MM. (In pNLB, *KpnI* resides 67 bp upstream of *lacZ* and *NdeI* resides 100 bp upstream of the *lacZ* stop codon.) The  
5 *IFN-MM* gene (SEQ ID NO:1) codes for human interferon-alpha-2B (IFN). To create pNLB-CMV-IFN-MM, the *HindIII/BlpI* insert of pNLB (containing *lacZ*) was replaced with the *HindIII/BlpI* insert of pNLB-Adapter-CMV-IFN-MM. This two step cloning was necessary because direct ligation of blunt-ended fragments into the *HindIII/BlpI* sites of pNLB yielded mostly rearranged subclones, for  
10 unknown reasons. A portion of the pNLB-CMV-IFN-MM vector sequence containing both the CMV promoter and *IFN-MM* is shown as SEQ ID NO:2.

Example 2. Production of a NLB-CMV-IFN-MM producing helper cell line.

Sentas and Isoldes were cultured in F10 (Gibco), 5% newborn calf serum  
15 (Gibco), 1% chicken serum (Gibco), 50 microgram/milliliter (ml) phleomycin (Cayla Laboratories) and 50 microgram/ml hygromycin (Sigma cat. # H-3274). Transduction particles were produced as described in Cosset et al., *Virology*, 1993, 65:2288-94, with the noted exceptions. Two days after transfection of the retroviral vector into  $9 \times 10^5$  Sentas, virus was harvested in fresh media for 6-16  
20 hours and filtered. All of the media was used to transduce  $3 \times 10^6$  Isoldes in 3 100 mm plates with polybrene added to a final concentration of 4 micrograms/ml. The following day the media was replaced with media containing 50 microgram/ml phleomycin, 50 microgram/ml hygromycin and 200 microgram/ml G418 (neomycin) (Sigma cat. # G-9516). After 10-12 days, single G418<sup>R</sup> colonies were  
25 isolated and transferred to 24-well plates. After 7-10 days, titers from each colony was determined by transduction of Sentas followed by G418 selection. Typically 2 out of 60 colonies gave titers at  $1-3 \times 10^5$ . Those colonies were expanded. The integrity of the CMV-IFN-MM expression cassette was confirmed by assaying for

interferon in the media of quail or chicken cells transduced with NLB-CMV-IFN-MM transduction particles.

Example 3. Production and concentration of VSV-G typed NLB-CMV-IFN-MM particles.

The retroviral particles were pseudotyped with the VSV-G envelope protein which enables receptor-independent adsorption of the particles (Burns et al., *Proc. Natl. Acad. Sci. USA*, 1993, 90:8033-7; Yee et al., *Methods Cell Biol.*, 1994, 43 Pt A:99-112). (Certain retroviral particles which were not pseudotyped with VSV-G had previously failed to lead to protein expression in the lumen through direct oviduct transgenesis.)

$4.5 \times 10^6$  Isolde helper cells which produce NLB-CMV-IFN-MM transduction particles (Isolde-NLB-CMV-IFN-MM) were plated in 2 to 6 75 cm<sup>2</sup> tissue culture treated polystyrene flasks (typically 6) (Falcon catalog # 3084) with complete F10 with hygromycin, phleomycin and neomycin added. (Typically 3 confluent 25 cm<sup>2</sup> flasks were harvested).  $4 \times 10^5$  Isolde-NLB-CMV-IFN-MM were plated per well in 1 6-well plate (Falcon catalog # 3046).

The next day, the 75 cm<sup>2</sup> flasks were transfected by mixing 40 micrograms of cesium chloride (CsCl) gradient-purified or Qiagen column-purified pCMV-G plasmid DNA and 3 mls of Optimem (Gibco-BRL) in a polystyrene tube and 46 microliters of DMRIE-C (Gibco-BRL) and 3 mls of Optimem in a second polystyrene tube. The plasmid pCMV-G expresses the G envelope glycoprotein of the vesicular stomatitis virus under control of the (CMV) promoter. The mixtures were combined, mixed and incubated for 15 minutes at room temperature. Meanwhile the flasks were washed twice with 12 mls of Optimem per wash. The six ml transfection mix was added to the flask followed by slow rocking for 4 hours in the 37°C CO<sub>2</sub> incubator. The mix was then aspirated, washed once with 12 mls of Optimem and refed with 20 mls complete F10 but with hygromycin and neomycin added (no phleomycin).

Typically two wells of the 6-well plate were transfected with pCMV-EGFP which expresses Enhanced Green Fluorescent Protein (EGFP; Clontech) under control of the CMV promoter. This served as a control for transfection efficiency. At the same time as the 75 cm<sup>2</sup> flask transfection was being carried out, 1 or 2  
5 wells of the 6-well plate were transfected by mixing 5 micrograms of purified pCMV-EGFP plasmid DNA (CsCl-purified or Qiagen-purified) and 0.25 mls of Opti-mem (Gibco-BRL) in a first polystyrene tube and 6 microliters of DMRIE-C (Gibco-BRL) and 0.25 mls of Opti-mem in a second polystyrene tube. The mixtures were combined, mixed and incubated for 15 minutes at room  
10 temperature. Meanwhile the wells were washed twice with 2 mls of Opti-mem per wash. The 0.5 ml transfection mix was added to the flask followed by slow rocking for 4 hours in the 37°C CO<sub>2</sub> incubator. Next, the mix was aspirated, washed once with 2 mls of Opti-mem and refed with 2 mls complete F10, but with hygromycin and neomycin added (no phleomycin).

15 24 hours later the 6-well plate was checked for transfection efficiency. (Transfection efficiency (with pCMV-EGFP) should be at least 30% and can be as high as 80% with DMRIE-C.)

24 hours post-transfection, the medium was aspirated from the 75 cm<sup>2</sup> flasks and the flasks were washed 2X with 4 mls F10 with no serum added. 12  
20 mls of F10 with 1% newborn calf serum (Gibco), 0.2% chicken serum and no drugs was added. 24 more hours later the medium was removed for filtration and new low serum F10 was added to the flasks. The harvested medium was filtered through a 0.45 micron filter (Gelman Sciences Acrodisc HT Tuffryn syringe filter, 0.45 micron) into a polypropylene tube (or a polyallomer quick-seal centrifuge  
25 tube, see below) and stored at 4°C until concentration. (Polystyrene tubes should not be used to store VSV-G typed particles. Polypropylene should be used instead.)

Particle supernatants were harvested from the flasks every 24 hours. VSV-G typed particle production peaked at 48-72 hours post-transfection (30-50% of

all particles were typed with VSV-G) and then trailed off to low levels by 120 hours (5-15% of all particles were typed with VSV-G).

At 120-144 hours post-transfection, the filtered supernatants were concentrated 10-100 fold as described in Allilioli et al., *Dev. Biol.*, 1994, 165:30-7, with the differences noted here. Harvested media was filtered by passage through a 0.45 um filter via a 60 ml syringe with an 18G needle attached to the filter, straight into 1 X 3 ½ inch polyallomer quick-seal centrifuge tubes (Beckman, 342414). The tubes were topped off with incomplete F10, capped with UV sterilized caps and sealed. Tubes were spun in a Beckman 60Ti rotor precooled to 4°C at 37,400 rpm (RCF = 98,977 g) for 30 minutes at high acceleration and deceleration. After the spin, some fluid was drained with a syringe and the top removed with a razor blade. All but 0.4 to 1.2 mls of the supernatant was removed without disturbing the pellet. The tube was then placed in a 37°C incubator so that the pellet was immersed and vigorously rocked for one hour to resuspend the pellet. With a micropipettor, the pellet was further triturated by pipetting up and down several times. Aliquots of the concentrated particles were stored at -70°C. (Typically, a loss of 50 % of the live virus occurred.)

Particles were titered on both Sentas and Isoldes to determine the titer of envA-typed particles and VSV-G typed particles. 2-3 x10<sup>5</sup> Isoldes and 2-3 x10<sup>5</sup> Sentas were plated per well in separate 6-well plates with no drugs added. One or two days later, an aliquot of the concentrated particles was thawed and diluted 1 in 500 with F10, complete. Each well was replaced with 2 mls of F10, complete, and 4 microliters of 1.0 mg/ml polybrene. 2 to 20 microliters of diluted particles were added to both a Senta well and an Isolde well. Twenty four hours later, the media was replaced with F10, complete, plus 200 microgram/ml G418 (neomycin). This media was changed every two days until distinct colonies formed. The number of colonies in a Senta well gave the titer, after correction for dilution factors, of both envA-typed particles and VSV-G typed particles. The number of colonies in an Isolde well gave the titer, after correction for dilution factors, of VSV-G typed

particles. It is assumed that most VSV-G typed particles also contained the envA-encoded envelope proteins and thus adsorbed to Sentas via the tv-a receptor or directly through the phospholipid membrane. Since Isoldes produced the envA-encoded envelope proteins, adsorption by envA-typed particles was blocked.

- 5 Therefore, particles adsorbed to Isoldes only if the particles contain the VSV-G envelope protein.

Example 4. Direct oviduct transgenesis (DOT) by surgical procedure

White Leghorn pullets which were 11 weeks old were used in the example.

- 10 Pullets which are between 10 and 20 weeks old can be used in the procedure. One to ten days prior to treatment, the pullets are preferably given daily dosages of diethylstilbestrol (DES, a potent form of estrogen) and/or progesterone and/or testosterone to stimulate proliferation of magnum cells. Doses will range from 0.1 mg to 24 mg per bird, depending of the weight of the bird. Typically, doses for a
- 15 1 kg hen are 1 mg of DES and 0.8 mg of progesterone, injected intramuscularly in a volume of 0.1 ml of 95% ethanol or sesame oil. Testosterone may be substituted for progesterone in some experiments. Additional hormone injections may be given the day of surgery and for several days after. The day before treatment, the pullets are taken off of their diet. In this example, 1 mg of DES and 0.8 mg of
- 20 progesterone per kg of pullet was injected daily for three days.

- On the morning of the fourth day, the magnum of the oviduct was accessed by surgical procedures. Pullets were anesthetized with a standard dose of isoflurane. Aliquots of the concentrated particles were thawed on ice. The magnum region of the oviduct was approached through a left lateral abdominal
- 25 incision. Laparoscopic grasping forceps were used to secure the oviduct during the injection. 0.5-0.6 mls of particles ( $1.6 \times 10^5$  to  $5 \times 10^5$  VSV-G typed particles from Example 3) were injected in two or three locations into the lumen of the magnum using a 1 ml syringe and 22G needle. The incision was sutured and the birds allowed to wake. The pullets were returned to their cages that afternoon



and given one last injection of DES and progesterone. Particle solutions remaining after injection were retitered on Isoldes and Sentas to confirm the viral titer. Six days later the same pullets were taken off their diet.

The next day the magnum was accessed through the same incision used for the injections. 0.5 ml of phosphate-buffered saline (PBS) was injected into the lumen. The lumen was gently massaged to mix the PBS with the lumen fluid. The sample was then removed with the same needle/syringe and placed on ice. After surgery the pullets were returned to their cages.

The 0.1 ml samples removed from the lumen of DOT-treated hens one week after transduction were assayed with an interferon-alpha-2B ELISA kit (Research Diagnostics, Inc.) using the high sensitivity protocol. The results are shown below in Table 1. Both DOT-treated hens had significant levels of human interferon in the lumen fluid. 0.1 ml samples from the media of chicken tubular gland cells two days after transfection with the indicated plasmids using the oviduct explant assay protocol (OEA) were also assayed (see Example 5, below).

**Table 1. Expression of interferon in the lumen of DOT-treated hens one week after treatment.**

Protocol	Sample	pg/ml
DOT	Pullet # 4039 lumen sample	137.1
DOT	Pullet # 4041 lumen sample	189.7
OEA	pCMV-EGFP transfected TGC cells ( <i>in vitro</i> ).	2.9
OEA	pCMV-IFN-MM transfected TGC cells ( <i>in vitro</i> ).	1108.8

Twelve weeks later, eggs were collected from the laying hens and assayed for the presence of interferon in the egg white. Egg white samples were removed from the eggs of untreated hens (treatment: "none") or from eggs of two hens that underwent direct oviduct transgenesis 1-2 weeks after those hens started to lay. 0.1 ml of egg white was assayed with an interferon-alpha-2B ELISA kit (Research

Diagnostics, Inc.) using the high sensitivity protocol. The amounts of interferon found in the eggs is shown in Table 2, below. One hen (#4039) had significant levels of interferon in the egg white as compared to egg white from untreated hens. The second hen (#4041), which did have interferon in the lumen one week after the DOT operation, did not have detectable levels of interferon in the white of her eggs. It is possible that the magnum cells of hen #4039 were transduced such that they remained in an active state after the magnum fully developed whereas the cells of hen #4041's magnum were sloughed off or silenced during development.

10

**Table 2. Deposition of interferon in the egg white of DOT-treated hens.**

Treatment	Date egg laid	Band #	pg/ml (ELISA)	pg/ml (Bioassay)
None	2/14/99	2369	31.2	0
None	2/11/99	1422	32.1	0
DOT with viral particles.	3/9/99	4039	179.0	291
DOT with viral particles.	3/10/99	4039	208.1	291
DOT with viral particles.	3/9/99	4041	32.2	ND
DOT with viral particles.	3/10/99	4041	23.1	ND

DOT: direct oviduct transgenesis; ND: not determined.

Bioactivity of human interferon in the egg white was determined by incubating human A-549 cells with dilutions of the egg white samples, challenging the cells with Menogvirus and counting viral-resistant plaques as described in Lewis, "Biological Assays for Interferons", in *Lymphokines and Interferons*, Clemens, ed., Oxford: IRL Press, pp.73-87, 1987. The interferon from hen #4039 was fully bioactive as determined by an anti-viral bioassay, confirming the expression of interferon in the hen's magnum.

15

Example 5. Oviduct explant assay (OEA).

The oviduct explant assay was performed as described in Sanders and McKnight, *Endocrinology*, 1985, 116:398-405, and Sanders and McKnight, *Biochemistry*, 1988, 27:6550-7, except that mature laying hens were used as the  
5 source of magnum tissue and DMRIE-C was used instead of calcium phosphate for the precipitation method. Supernatants from cells transfected with the expression cassette pCMV-IFN-MM or pCMV-EGFP (see Example 3, above) were collected 48 hours post-transfection and assayed by ELISA. pCMV-IFN-MM was the same expression cassette that was inserted into pNLB-CMV-IFN-  
10 MM (see Example 1, above) pCMV-EGFP served as a negative control plasmid. 0.1 ml of sample from the transfected cells was assayed with an interferon-alpha-2B ELISA kit (Research Diagnostics, Inc.) using the high sensitivity protocol. The results are shown in Table 1 above.

15 All documents cited in the above specification are herein incorporated by reference. Various modifications and variations of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as  
20 claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in the art are intended to be within the scope of the following claims.

We claim:

1. A method for preparing a transgenic avian which expresses an exogenous protein substantially only in its oviduct, comprising:
  - 5 delivering a nucleic acid expression cassette directly to the oviduct of an immature avian, wherein the nucleic acid expression cassette comprises (i) a promoter which is active in the avian oviduct and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter.
- 10 2. A method for preparing a transgenic avian which lays eggs containing exogenous protein, comprising:
  - preparing a transgenic avian which expresses an exogenous protein substantially only in its oviduct according to the method of claim 1; and
  - 15 allowing the immature avian to grow to maturity,wherein the exogenous protein is secreted into the oviduct lumen of the mature avian and deposited into eggs laid by the avian.
- 20 3. The method of claim 1, further comprising a first step of administering a steroid hormone treatment to the immature avian.
4. The method of claim 1, wherein the step of delivering the expression cassette comprises a method selected from laparoscopy, transcloacal catheterization, and opening the abdominal wall.
- 25 5. The method of claim 1, wherein delivery of the expression cassette is aided by a delivery vehicle selected from the group consisting of an adenovirus, a retrovirus, and a liposome.

6. The method of claim 5, wherein the retrovirus is derived from the avian leukosis virus (ALV) or the vesicular stomatitis virus (VSV).
7. The method of claim 5, wherein the retrovirus is an ALV-based retrovirus which has been pseudotyped with the G envelope glycoprotein of the vesicular stomatitis virus.
8. The method of claim 5, wherein the retrovirus comprises an oncogene or growth factor gene in its genome.
9. The method of claim 8, further comprising coexpression of an inhibitor of apoptosis.
10. The method of claim 5, wherein the delivery vehicle comprises a retrovirus and the method further comprises the additional step of coinfecting the oviduct with a retrovirus that expresses an oncogene or growth factor.
11. The method of claim 1, wherein said delivery step comprises
- (a) preparing helper cells which produce a virus carrying the expression cassette; and
  - (b) injecting the helper cells into the oviduct.
12. The method of claim 1, wherein the immature avian is an immature chicken, turkey, duck, goose, or quail.
13. The method of claim 12, wherein the immature avian is an immature chicken which is from about 10 to about 20 weeks old.

14. The method of claim 1, wherein the nucleic acid expression cassette codes for a protein selected from the group consisting of human growth hormone, interferon,  $\beta$ -casein,  $\alpha$ -1 antitrypsin, antithrombin III, collagen, factors VIII, factor IX, factor X, fibrinogen, hyaluronic acid, insulin, lactoferrin, protein C, erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), tissue-type plasminogen activator (tPA), feed additive enzymes, somatotropin, and chymotrypsin.
15. The method of claim 14 wherein the nucleic acid expression cassette codes for interferon  $\alpha$ -2b.
16. A method for preparing a transgenic avian which lays eggs containing exogenous protein, comprising:
- delivering a nucleic acid expression cassette directly to the oviduct of a mature avian, wherein the nucleic acid expression cassette comprises (i) a promoter which is active in the avian oviduct and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter,
- wherein the exogenous protein is expressed in the avian's oviduct, secreted into the lumen of the oviduct and deposited into eggs laid by the avian.
17. A method for producing an avian egg that contains exogenous protein, comprising:
- delivering a nucleic acid expression cassette directly to the oviduct of an immature avian, wherein the nucleic acid expression cassette comprises (i) a promoter which is active in the avian oviduct and (ii) a nucleic acid sequence which is operably linked to the promoter and which codes for an exogenous protein; and
- allowing the immature avian to grow to maturity,

wherein said exogenous protein is expressed in the oviduct, secreted into the lumen of the oviduct, and deposited into eggs laid by the avian.

18. A method for producing a protein, comprising:
- 5                   producing an avian egg that contains exogenous protein according to the method of claim 17; and
- isolating said exogenous protein from said egg.
19. A method for testing the efficiency of the expression of a transgene in an
- 10   avian oviduct, comprising:
- delivering a nucleic acid expression cassette directly to the oviduct of an immature avian, wherein the nucleic acid expression cassette comprises (i) a promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter; and
- 15                   assaying for the presence or amount of the exogenous protein in the lumen of the oviduct.
20. A method for testing the ability of a promoter to facilitate expression of a transgene in an avian oviduct, comprising:
- 20                   delivering a nucleic acid expression cassette directly to the oviduct of an immature avian, wherein the nucleic acid expression cassette comprises (i) the promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter; and
- assaying for the presence or amount of the exogenous protein in the
- 25                   fluid of the lumen of the oviduct.
21. A method for screening a preparation of viral particles for deleterious mutations, comprising:

delivering the viral particles from a single preparation to the oviduct of an immature avian, wherein the viral particles contain nucleic acid expression cassettes comprising (i) a promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter; and

assaying for the presence or amount of the exogenous protein in the lumen of the oviduct.

22. A method for testing the suitability of a transgene for expression in an avian oviduct or for secretion of its expression product into the lumen of the oviduct and into the eggs of an avian, comprising:

delivering a nucleic acid expression cassette directly to the oviduct of an immature avian, wherein the nucleic acid expression cassette comprises (i) a promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter;

allowing said immature avian to grow to maturity; and

assaying for the presence or amount of the exogenous protein in the egg white of eggs laid by the avian.

23. An avian having a transgene encoding an exogenous protein substantially only in its oviduct, wherein the avian secretes the exogenous protein expressed by the transgene into its eggs.

24. An avian having an exogenous protein substantially only in its oviduct, wherein the avian secretes the exogenous protein into its eggs.

25. The avian of claim 24, wherein said exogenous protein comprises human interferon.



26. An intact avian egg containing protein exogenous to the avian egg.
27. The egg of claim 26, wherein said protein comprises human interferon.
- 5 28. An avian leukosis virus pseudotyped with the G envelope glycoprotein of the vesicular stomatitis virus.
29. An isolated polynucleotide comprising the sequence set forth in SEQ ID NO:1, the complement thereof, an at least 12 nucleotide-long fragment thereof, or  
10 a sequence that hybridizes thereto, wherein said polynucleotide is not a fragment of an interferon alpha-2b gene found in nature.
30. An expression vector comprising:
- (i) the polynucleotide of claim 29; and
  - 15 (ii) a promoter, wherein said promoter is operably linked to said polynucleotide.

## SEQUENCE LISTING

<110> Ivarie, Robert  
 Harvey, Alex J.  
 Murphy Jr., George F.  
 Rapp, Jeffrey C.

<120> Direct Oviduct Transgenesis

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 <213> Homo sapiens

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>C12N 15/00, A01K 67/027, C07K 14/56,</b> <b>C12N 15/86, 7/04, 7/01, 15/21, G01N</b> <b>33/53</b>		<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 00/11151</b> <b>(43) International Publication Date:</b> 2 March 2000 (02.03.00)
<b>(21) International Application Number:</b> PCT/US99/19393 <b>(22) International Filing Date:</b> 25 August 1999 (25.08.99)  <b>(30) Priority Data:</b> 09/139,902 25 August 1998 (25.08.98) US  <b>(71) Applicants:</b> UNIVERSITY OF GEORGIA RESEARCH FOUNDATION, INC. [US/US]; The Boyd Graduate Studies Research Center, Athens, GA 30602-7411 (US). AVIGENICS, INC. [US/US]; 845 Oak Grove Avenue #220, Menlo Park, CA 94025 (US).  <b>(72) Inventors:</b> IVARIE, Robert; 54 Jackson Street, Watkinsville, GA 30677 (US). HARVEY, Alex, J.; 188 Sunset Drive, Athens, GA 30606 (US). MURPHY, George, F., Jr.; 1723 Santa Cruz Avenue, Menlo Park, CA 94025 (US). RAPP, Jeffrey, C.; 48 Third Street, Watkinsville, GA 30677 (US).  <b>(74) Agents:</b> CHOW, Y., Ping et al.; Heller Ehrman White & McAuliffe, 525 University Avenue, Palo Alto, CA 94301-1900 (US).		<b>(81) Designated States:</b> AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), DM, EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>  <b>(88) Date of publication of the international search report:</b> 15 June 2000 (15.06.00)	
<b>(54) Title:</b> DIRECT OVIDUCT TRANSGENESIS			
<b>(57) Abstract</b>  Methods for preparing transgenic avians which express exogenous protein substantially only in their oviducts are disclosed. Each of the methods comprises delivering nucleic acid expression cassettes directly to the oviducts of the avians. The exogenous protein expressed by the expression cassette is secreted into the lumen of the avian oviduct and secreted into the eggs of the transgenic avians. Methods for preparing eggs which contain exogenous protein, such as human interferon, and methods for the production of proteins are also disclosed. The methods for direct oviduct transgenesis may also be used to assess the suitability of expression cassettes or exogenous proteins for expression in the avian oviduct.			



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DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

# INTERNATIONAL SEARCH REPORT

		International Application No PCT/US 99/19393	
<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 7 C12N15/00 A01K67/027 C07K14/56 C12N15/86 C12N7/04 C12N7/01 C12N15/21 G01N33/53			
According to International Patent Classification (IPC) or to both national classification and IPC			
<b>B. FIELDS SEARCHED</b>			
Minimum documentation searched (classification system followed by classification symbols) IPC 7 A01K C12N C07K			
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched			
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)			
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>			
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	
X	MURAMATSU, T. ET AL.: "Gene gun-mediated in vivo analysis of tissue-specific repression of gene transcription driven by the chicken ovalbumin promoter" MOLECULAR AND CELLULAR BIOCHEMISTRY, vol. 185, no. 1-2, 6 August 1998 (1998-08-06), pages 27-32, XP002095226	1,4,12	
Y	the whole document --- -/--	1-18, 22-25	
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.			
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "Z" document member of the same patent family			
Date of the actual completion of the international search  1 February 2000		Date of mailing of the international search report  19. 04. 00	
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3018		Authorized officer  CHAMBONNET, F	

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/19393

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OCHIAI H, PARK HM, NAKAMURA A, SASAKI R, OKUMURA JI, MURAMATSU T: "Synthesis of human erythropoietin in vivo in the oviduct of laying hens by localized in vivo gene transfer using electroporation." POULT SCI., vol. 77, no. 2, February 1998 (1998-02), XP000863530	1,4,12, 14
Y	cited in the application the whole document	1-18, 22-25
Y	--- WO 97 47739 A (MACARTHUR WILLIAM C ;UNIV MICHIGAN (US); GENWORKS L L C (US)) 18 December 1997 (1997-12-18) cited in the application claims 18-22	2,5,6, 16-18, 22-25
P,Y	--- WO 99 19472 A (AVIGENICS ;UNIV GEORGIA (US)) 22 April 1999 (1999-04-22) claims 19-29,35-39 -----	1-18, 22-25

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/ 19393

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See additional sheets

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-18, 22-25

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 99/19393

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-18 22-25

A method for preparing a transgenic avian which expresses an exogenous protein substantially only in its oviduct, or which lays eggs containing exogenous protein, comprising: delivering a nucleic acid expression cassette directly to the oviduct of an immature avian, wherein the nucleic acid expression cassette comprises (i) a promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter; said transgenic avian.

2. Claims: 19-20

Methods for testing the efficiency of or the ability of a promoter to facilitate expression of a transgene in an avian, oviduct comprising : delivering a nucleic acid expression cassette directly to the oviduct of an immature avian, wherein the nucleic acid expression cassette comprises (i) a promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter; and assaying for the presence or amount of the exogenous protein in the lumen of the oviduct.

3. Claim : 21

A method for screening a preparation of viral particles for deleterious mutations comprising: delivering the viral particles from a single preparation to the oviduct of an immature avian, wherein the viral particles contain nucleic acid expression cassettes comprising (i) a promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter; and assaying for the presence or amount of the exogenous protein in the lumen of the oviduct

4. Claims: 26-27

An intact avian containing protein exogenous to the avian egg.

5. Claim : 28

An Avian Leukosis Virus pseudotyped with the G envelope glycoprotein of the Vesicular Stomatitis Virus

6. Claim : 29 30

An isolated polynucleotide comprising the sequence set in SEQ ID NO:1, the complement thereof, an at least 12

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 99/19393

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

nucleotide-long fragment thereof, or a sequence that hybridizes thereto, wherein said polynucleotide is not a fragment of an interferon alpha-2b found in nature; an expression vector comprising said nucleotide and a promoter linked thereto.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/19393

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9747739 A	18-12-1997	AU 3479997 A	07-01-1998
		CA 2257871 A	18-12-1997
		EP 0960194 A	01-12-1999
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WO 9919472 A	22-04-1999	AU 1189999 A	03-05-1999
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